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Substitute Form PTO 1390 U.S. Department of Commerce Patent and Trademark Office	Attorney's Docket Number: 50015/013001
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	U.S. Application Number: [**US SERIAL NUMBER**]

INTERNATIONAL APPLICATION NUMBER	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/01322	22 January 1998	22 January 1997

TITLE OF INVENTION:	*IN VIVO* APOPTOSIS SCREENING
APPLICANTS FOR DO/EO/US:	Lee L. Rubin, Christine de Leon Gatchalian

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1.	X	This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2.		This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3.	X	This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4.	X	A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5.	X	A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
a.		is transmitted herewith (required only if not transmitted by the International Bureau).
b.		has been transmitted by the International Bureau.
c.	X	Is not required, as the application was filed with the United States Receiving Office (RO/US).
6.		A translation of the International Application into English (35 U.S.C. 371(c)(2).
7.		Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
a.		are transmitted herewith (required only if not transmitted by the International Bureau).
b.		have been transmitted by the International Bureau.
c.		have not been made; however, the time limit for making such amendments has NOT expired.
d.		have not been made and will not be made.
8.		A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9.		An (unexecuted) oath or declaration of the inventors (35 U.S.C. 371(c)(4)).

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10.		A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5).
11.		An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12.		An assignment for recording. A separate cover sheet in compliance with 37 3.28 and 3.31 is included.
13.		A FIRST preliminary amendment.
		A SECOND or SUBSEQUENT preliminary amendment.
14.		A substitute specification.
15.		A change of power of attorney and/or address letter.
16.	X	Other items or information: Postcard; Copy of published PCT application (WO 98/31787)

17.		The following fees are submitted:	
		BASIC NATIONAL FEE (37 CFR 1.492(A)(1)-(5)):	
		Search Report has been prepared by the EPO or JPO $ 930.00	
	X	International preliminary examination fee paid to USPTO (37 CFR 1.482) $ 670.00	$670.00
		No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $ 790.00	
		Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $ 1070.00	
		International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $ 98.00	

			ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 670.00	
Surcharge of $130 for furnishing the oath or declaration later than □ 20 OR □ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	31 - 20 =	11	x $22.00	$ 242.00	
Independent claims	5 - 3 =	2	x $82.00	$ 164.00	
Multiple dependent claims (if applicable)			+ $270.00	$	
			TOTAL OF ABOVE CALCULATIONS =	$ 1,076.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed with this request (Note 37 CFR 1.9, 1.27, 1.28).				$	
			SUBTOTAL =	$ 1,076.00	
Processing fee of $130.00 for furnishing the English translation later than □ 20 OR □ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
			TOTAL NATIONAL FEE =	$ 1,076.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property. +				$	
			TOTAL FEES ENCLOSED =	$ 1,076.00	

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	Amount to be refunded	$
	charged	$

a.	X	A check in the amount of $ 1,076.00 to cover the above fees is enclosed.
b.		Please charge my Deposit Account No. 03-2095 in the amount of $ [**.**] to cover the above fees.
c.	X	The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 03-2095. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO: ~~Paul T. Clark~~ ~~Clark & Elbing LLP~~ ~~176 Federal Street~~ Boston, MA 02110-2214 Telephone: 617-428-0200 Facsimile: 617-428-7045	Susan M Michaud Signature Susan M. Michaud Paul T. Clark Reg. No. 42,885 Reg No. 30,162

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IN VIVO APOPTOSIS SCREENING

Background of the Invention

This invention relates to apoptosis, which is associated with physiological or programmed cell death (PCD). Apoptosis occurs in embryonic development, hormone deprivation of endocrine or other hormone-dependent or sensitive cells, cells responding to mild thermal or metabolic stress, and normal tissue turnover. Compounds which affect PCD (either accelerating or inhibiting the process) are potentially useful as therapeutics to treat a wide range of medical disorders, including cancer, AIDS, autoimmune disorders such as rheumatoid arthritis, and neurodegenerative diseases such as multiple sclerosis.

Cell death in many types of cells including neurons can be thought of as a three-step process. The first step is the transmission of information about the status of the cell from outside the cell to the cytoplasm, or from the cell membrane to the nucleus. This information may follow the appearance of an apoptosis-inducing factor such as the Fas ligand, or the disappearance of a survival-promoting factor, such as nerve growth factor (for some types of neurons). The second step is gene transcription and translation into protein. This second step can be blocked by compounds such as actinomycin D or cycloheximide, compounds which block transcription or translation. In this manner, these agents block some types of apoptosis. In the third step, the effectors of cell death are activated; these effectors include (in many types of cells) cysteine proteases which cleave after aspartic acid residues; these agents

are now termed caspases. Caspase inhibitors can interrupt the programmed cell death chain of processes, by blocking this third step.

Summary of the Invention

We have developed novel methods for screening potential apoptosis-affecting compounds in an intact animal. One aspect of the invention features a method of testing a compound for the ability to affect cell death. This method includes: a) providing an osteichthes embryo which is translucent or transparent (i.e., optically clear), b) contacting the compound with the clear embryo, and c) visually observing the pattern or extent of cell death in the embryo. The visual observation can be accomplished by a variety of methods known to those in the art of cytology, including labeling cells which undergo programmed cell death or apoptosis in the living embryo for visualization microscopically. For example, one method of labeling is carried out by terminal deoxynucleotide transferase dUTP nick labeling.

Another aspect of the invention features a method which includes the steps of: a) providing an animal (e.g., a vertebrate, such as a fish, and preferably a zebrafish, *Danio rerio*) which, at an embryonic stage, has been contacted with an agent which increases apoptosis in cells of the animal, b) contacting the animal with the test compound, and c) determining whether the compound affects cell death in the animal. For example, the contacting step b) is carried out with the animal at an embryonic stage. The determining step c) may include determining whether the compound affects cell death in a Rohon-Beard neuron. The determining step c) may include determining whether the compound decreases or inhibits apoptosis (programmed cell death) in a Rohon-Beard neuron that has been contacted with an agent which increases apoptosis. A compound which decreases or inhibits apoptosis is said to have

rescued a cell. The determining step c) can also include using an antibody to label a cell, such as a Rohon-Beard cell, which undergoes cell death.

The invention also includes a test vertebrate embryo useful for screening compounds for the ability to affect cell death. The test embryo is prepared by a) providing a vertebrate embryo (e.g., a zebrafish), and b) contacting the embryo with an agent (e.g., stauro-sporine) which increases apoptosis in cells of the animal. This test embryo can be used in the methods disclosed herein for testing a compound for the ability to affect cell death.

Furthermore, the test embryo of the invention can also be used in a variety of methods for obtaining information on cellular processes. The cellular process which is investigated is selected from the group consisting of: a) neuronal cell function, b) neuronal connectivity, c) cell development, d) tissue development, and e) organ development. This method includes a) providing at least two embryos, namely a test zebrafish embryo and a control zebrafish embryo, b) subjecting a test embryo to test conditions, and c) visually observing differences in cells of the test and control embryos. The control embryo is treated such that the differences in visual observation result from application of the test conditions.

Test conditions include mutagenesis-inducing conditions (e.g., radiation or chemical agents), apoptosis-inducing or increasing agents, apoptosis-inhibiting or decreasing compounds, labeling or staining of one or more types of cells, marker compounds which label or indicate the present of a metabolite, and ligands for a receptor. In one embodiment, the test and control zebrafish embryos are pre-treated with an agent which affects programmed cell death. In another embodiment, the test conditions include a test compound (for example, either a cell death inhibitor, such as a caspase inhibitor, or a compound which increases cell death), and the observing step includes

observing whether the test compound inhibits cell death in the test embryo, in other words, whether the test compounds rescues the test embryo. The agent can be a protein kinase inhibitor, such as staurosporine. The observing step can include observing neurons of the embryos for an interval of time sufficient to determine whether neurons in the test embryo which are saved from cell death develop or function normally. The observing step can include observing or comparing Rohon-Beard neurons in the test and control embryos. The invention also features a method for testing a compound for the ability to affect expression of a gene whose expression affects cell death, said method comprising the steps of: (a) providing an osteichthes test embryo which is translucent or transparent, wherein the gene is expressed in the embryo, wherein the gene either is not normally expressed in the osteichthes (embryo or adult), or is normally expressed in the osteichthes (embryo or adult) at a lower level than in the test embryo, (b) contacting the compound with the embryo, and (c) visually observing the pattern or extent of cell death in the embryo. The osteichthes embryo can be a zebrafish embryo. The gene can be a eukaryotic gene encoding a protein which inhibits cell death, such as a gene which encodes bcl-2, and is over-expressed in the embryo.

The invention in part is based on the observation that cells in the zebrafish embryo undergo apoptosis during normal development. According to the invention, the dying cells can be identified by simply viewing the whole live embryo (e.g., using Nomarski optics, or by a vital stain such as acridine orange). Alternatively, the dying cells can then be viewed and analyzed histologically, by staining the entire embryo using a method (the TUNEL method, explained in detail below), which detects DNA in the process of fragmenting during cell death. The pattern of TUNEL-positive cells at about 24 hours of development is easily discernable and very reproducible. The

present studies have indicated that some of the dying cells are neurons, including Rohon-Beard sensory neurons, and thus the method provides an important tool for studying neuronal apoptosis.

Zebrafish are well suited for use in the method of the invention because of their rapid development, large brood size, external fertilization and, most importantly, the optical clarity of their embryos. Because the embryo is clear, apoptotic cells can be detected under the light microscope as highly refractive bodies, or stained using vital dyes such as acridine orange.

The amount of normal cell death in the developing fish embryo, as in other vertebrate embryos, is relatively small at any point in time. According to another aspect of the invention, the amount of cell death in these embryos can be greatly increased by briefly exposing the live embryos to a protein kinase inhibitor such as staurosporine. The experiments demonstrated that exposure of embryos to micromolar amounts of staurosporine produced large numbers of TUNEL-positive cells. The number of dying cells was so large that they could easily be observed by microscopy, even without TUNEL staining. Cell death was so extensive that entire structures, e.g., the caudal tail region, were affected in a manner which could be observed microscopically.

Embryos can be treated with agents which inhibit apoptosis, e.g., caspase inhibitors, and the "saved" neurons can then be examined to determine whether they develop or function normally. Neural connectivity can also be observed using the system of the invention, as can cell, tissue, and embryonic development.

The invention can also be used (a) to test the effects of expression of a foreign gene whose expression ordinarily affects cell death, or (b) to screen for inhibitors of either a foreign gene whose expression ordinarily affects cell death or an endogenous gene that affects cell death. The method involves the

steps of: (a) providing an osteichthes (preferably zebrafish) test embryo which is translucent or transparent, and in which the cell death-affecting gene is expressed; the gene either is one which is not normally expressed in the species of which the embryo is a member, or is normally expressed at lower levels, and is over-expressed in the test embryo. The test compound is contacted with the test embryo, and changes in the pattern or extent of cell death in the embryo brought about by the compound indicate its effect on cell death. Compounds which inhibit cell death-blocking compounds are useful, e.g., as anti-tumor adjuvant therapeutics.

Any of the known genes which express proteins which inhibit or accelerate cell death can be used; one example is the bcl-2 gene, the overexpression of which can be expected to block apoptosis. The embryo can be caused to express or over-express the cell death-affecting gene either via a transgenesis (the gene is inserted into embryo by standard microinjection techniques), or cells which have been transfected *ex-vivo* with the gene can be transplanted into the embryo. The human sequence for bcl-2 was published in Cleary, M.L. and Sklar, J., "Cloning and structural analysis of cDNAs for bcl-2 and hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation," Cell 47:19-28, 1986. The murine bcl-2 sequence was published in Negrini et al., "Molecular analysis of mbcl-2; Structure and expression of the murine gene homologous to the human gene involved in follicular lymphoma," Cell 49:455-463, 1987; and Nunez et al., "Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines," J. Immunol. 144:3602-3610, 1990.

The invention offers ease of use compared to other vertebrate embryonic systems (such as rodent or avian), while the process of cell death is similar or identical. Thus, compounds identified according to the invention

which are effective anti-apoptotic agents are likely to be effective in mammalian systems as well. A further advantage of the invention is that the embryos are treated while they are still alive and developing, and it is therefore possible to determine if cells that are prevented from dying develop normally, an important consideration in screening anti-cell death drug candidates.

Other features and advantages of the invention will be apparent from the detailed description thereof, and from the claims.

Detailed Description of the Invention

Embryos

The embryos used in the methods of the invention preferably are clear and large enough for easy microscopic visualization . A number of osteichthes (bony fish) species are suitable, e.g., Medaka, Giant rerio. The preferred species is *Danio rerio*, the zebrafish, which has large, clear, easily-visualized embryos, and which reproduces in large numbers.

Cell Death Inhibition Assay

The assay is carried out as follows. Ten zebrafish embryos, at 90% epiboly (about 9 hours), are placed in a tank containing standard fish H_2O (60 mg Instant Ocean/liter distilled water) and varying concentrations (0.001-1000 μM) of test compound. The embryos are incubated with the test compound overnight (until they reach about 22 hours of development), and examined *in vitro* and then prepared as follows for histologic examination.

The embryos are either acridine orange labelled or dechorionated and fixed in 4% paraformaldehyde made up in PBS. They can then be viewed with Nomarski optics or processed for TUNEL.

Acridine Orange Staining

Embryos of different ages are collected and incubated for 15-20 minutes in 5μg/ml acridine orange (Sigma). The embryos are then

anaesthetized and observed under a microscope. Acridine orange-positive apoptotic cells are clearly visible under fluorescent illumination. They are then photographed and counted.

TUNEL Staining

The acronym TUNEL stands for terminal deoxynucleotide transferase (TdT) dUTP nick labelling, a method which detects DNA fragmentation which is characteristic of dying cells. In this method, terminal deoxynucleotide transferase DNA polymerases target the multitude of new 3'OH ends generated by DNA fragmentation in both early stage and morphologically identifiable nuclei and apoptotic bodies. TdT polymerases add digoxigenin-dUTP to the 3'OH ends of the PCD fragmented DNA, which can then be detected by anti-digoxigenin alkaline phosphase conjugate, and stained with substrate.

In summary, the TUNEL staining method is carried out as follows:

Embryos are fixed and washed in PBT buffer. They are then treated with proteinase K, washed, and postfixed in paraformaldehyde. They are rinsed, fixed in methanol/acetic acid, rinsed again, and then subjected to the terminal transferase reaction. Embryos are incubated with terminal deoxytransferase (TdT) using reagents and conditions provided in the Apoptosis Detection Kit supplied by Oncor, Inc. Enzyme incubation is overnight at 37°C . The reaction is stopped and the embryos are rinsed in PBT. For detection of labelled DNA, embryos are incubated in sheep anti-digoxigenin antibody conjugated to alkaline phosphatase. An alkaline phosphatase substrate X-phosphate/NBT in an appropriate buffer is added and incubation continues for 15 minutes. The reaction is stopped, embryos are fixed, cleared, mounted, and viewed with Nomarski optics.

The detailed TUNEL protocol is as follows:

Fixation

1. Dechorionate embryos and fix in 4% paraformaldehyde/PBS for 1 hour at room temperature. Wash 3 x 5 minutes in PBS. Embryos can be stored in methanol at 4°C overnight.

Permeabilization

1. Rehydrate by careful washes in 75% methanol + 25% PBT (1 x PBS, 0.1% Tween 70); 50% methanol; 50% PBT; 75% methanol; 75% PBT for 5 minutes each.

2. Wash 3 x for 5 minutes in PBT.

3. Incubate embryos in Proteinase K (10 µg/ml in PBS) at room temperature 20 minutes for post 16 hours. Wash 2 x for a few seconds in PBT.

4. Postfix embryos in 4% paraformaldehyde/PBS for 20 min. at room temperature.

5. Wash 5 x 5 minutes in PBT.

6. Postfix embryos for 10 minutes at -20°C with prechilled (-20°C) Ethanol:Acetate +2:1.

7. Wash 3 x 5 minutes in PBT at room temperature.

Terminal Transferase Reaction

1. Incubate embryos for 1 hour at room temperature in 75 µl (1 drop) equilibration buffer, reaction buffer and TdT enzyme are provided in the ApopTag In situ Apoptosis Detection Kit-Peroxidase, Oncor, Inc. For preparation of working strength TdT enzyme, mix the reaction buffer (S7105) with the TdT enzyme (S7107)=2:1 and add Triton X 100 to a final concentration of 0.3%).

2. Take off as much equilibration buffer as possible and add small volume of working strength TdT enzyme (The reaction worked already with as little as 17 µl working strength TdT enzyme). Incubate overnight at 37°C.

Stop/Wash

1. Stop reaction by washing in working strength stop/wash buffer (prepare working strength stop/wash buffer by mixing 1 ml stop/wash buffer (S7100-4) with 17 ml distilled water) for 3 hours at 37°C.

2. Wash 3 x 5 minutes in PBT.

Detection

1. Block with 2 mg/ml BSA, 5% sheep serum in PBT for a minimum of 60 minutes (or use 4% BSA, 5% non-fat dry milk, 10% horse serum).

2. Incubate embryos for 2 hours at room temperature (or overnight at 4°C) in a 1/2000 dilution of preabsorbed sheep anti-digoxgenin-alkaline phosphatase conjugated Fab fragments.

3. Wash overnight with 2 mg/ml BSA in PBT with at least 4 changes of blocking buffer.

4. Equilibrate 3 x 5 minutes in freshly prepared NTMT buffer (0.1 M Tris-HCl pH 9.5; 50 mM $MgCl_2$; 0.1M NaCl; 0.1% Tween 20).

5. Perform color reaction with X-phosphate/NBT in NTMT on shaker in dark (4.5 μl of 75 mg/ml NBT in dimethylformamide and 3.5 μl of 50 mg/ml X-phosphate in dimethylformaldehyde in 1 ml NTMT buffer) for 15 minutes.

6. Stop reaction with washes in PBT.

Clearing and Mounting

1. Fix embryos in 4% paraformaldehyde/PBS for 30 minutes at room temperature.

2. Wash in PBT.

3. Clear and mount in glycerol - 30% - 50% - 70%.

4. Document with Nomarski optics.

Other staining methods which detect DNA fragmentation characteristic of dying cells can be used in place of TUNEL staining, e.g., propidium iodide or Hoechst 33342 dye incubation followed by examination under fluorescence optics to detect condensed chromatin. Extraction of DNA is followed by resolution on gels to detect DNA "laddering" into nucleosome-sized fragments of about 180 bp.

Programmed Cell Death in Normal Embryos

TUNEL staining was used to identify the pattern of PCD occurring in various stages of normally-developing zebrafish. Embryos 30% epiboly to 24 hours were studied every hour, and embryos 24 hours to 48 hours were studied every two hours.

Diffuse PCD was observed in most regions during development, with concentrated regions of PCD which were localized spatially and temporally. The earliest PCD was detected in a few cells at 75% epipboly. Up to 12 somites, there is diffuse, seemingly random PCD, which then begins to concentrate toward the brain and tailbud. From 19 hours, a localized pattern of PCD was found in the lens and cornea of the eye, the otocyst, the cloacal opening, the olfactory placode, and portions of the nervous system, including the dorsoventral spinal cord.

Diffuse apoptosis is believed to occur amidst tightly packed cells in order to allow their free movement during periods of gradual morphogenesis. Highly localized concentrations of PCD may permit more radical morphogenesis. For example, localized cell death in the nervous system may clear the way for outgrowing axons; concentrated PCD in the olfactory placode coincides with the time that axons are exiting the placode and growing toward the telencephalon.

PCD Increase with Kinase Inhibitors and Decrease with Caspase Inhibitors

The protein kinase inhibitor staurosporine is used at a concentration of between 10 μM and 100 μM. Embryos at the 22 hour stage are incubated for 120 minutes, washed in PBS, and either labelled with acridine orange or fixed and viewed in Nomarski optics or processed for TUNEL.

EXAMPLE 1

An assay according to the invention was carried out with staurosporine-pretreated zebrafish embryos, using, as a test compound, a tripeptide inhibitor of ICE-like proteases, carboxybenzoyl Val-Ala-Asp fluoromethylketone (zVADfmk). A control, carboxybenzoyl Phe-Ala-fluoromethylketone (zFAfmk), did not prevent apoptosis in either staurosporine-pretreated embryos, or untreated embryos. The results demonstrated that ICE-like proteases are likely to be involved in mediating apoptosis, within the nervous system and perhaps in other organ systems as well.

TABLE 1

Conditions	Normal PCD	Sts-induced PCD
untreated	25 ± 3.15	455 ± 132
+zVADfmk	10 ± 2.76	31 ± 3.47
+zFAfmk	31 ± 2.79	322 ± 64.27

Table 1. Number of TUNEL-positive cells in the dorsal half of 26-28 hour old zebrafish embryos spanning a 10-somite length. Values expressed as means ± SEM of 5 embryos per condition.

EXAMPLE 2

Rohon-Beard neurons are sensory neurons which undergo apoptosis. HNK-1 is a cell surface molecule which is predominantly expressed by Rohon-Beard neurons. Zebrafish embryos were double stained with TUNEL to mark those cells undergoing apoptosis and with HNK-1 antibody to identify Rohon Beard neurons. Standard procedures were used for antibody labeling embryos ranging from 12 somites to 48 hours. Embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 4 hours, washed in PBS, incubated for 3 hours in PBS plus 3% goat serum and 0.1% Triton-X-100 (Sigma), and incubated overnight in a solution of monoclonal HNK-1 antibody (Sigma) diluted 1:1000. This was followed by washing in PBS plus 0.1% Triton (PBST) for at least one hour and incubating overnight in HRP-conjugated goat anti-mouse IgM diluted in PBST plus 1% goat serum. Embryos were washed as before and bound antibody was detected using diaminobenzidene (DAB) as the chromogen. Double labeling cells with HNK-1 antibody and TUNEL involved first HNK-1 antibody staining followed by TUNEL using the methods sequentially as already described.

Color photographs of zebrafish spinal cord demonstrated that, in an apoptosis assay, Rohon-Beard neurons were rescued by an apoptosis inhibitor compound such as zVADfmk (TUNEL-negative). In contrast, neurons not exposed to the inhibitor compound were TUNEL positive, indicating cell death. This example demonstrated that *in vivo* screening methods disclosed herein can reliably identify cells which are undergoing cell death, and, by substituting the known apoptosis inhibitor with a test compound or mixture of test compounds, also identify compounds which affect cell death, for example, apoptosis inhibitors.

Other Embodiments

Based on the description and examples above, and the claims below, the essential features and advantages of the present invention can be ascertained. Without departing from the spirit and scope of this disclosure, further various modifications or substitutions can be made and are also within the invention.

Claims

1. A test vertebrate embryo for screening compounds for the ability to affect cell death, said embryo being prepared by the process of:

a) providing a vertebrate embryo, and

b) contacting said embryo with an agent which increases apoptosis in cells of said animal.

2. The embryo of claim 1, wherein said agent is staurosporine.

3. The embryo of claim 1, wherein said vertebrate is a zebrafish.

4. A method of testing a compound for the ability to affect cell death, said method comprising the steps of:

a) providing an animal which, at an embryonic stage, has been contacted with an agent which increases apoptosis in cells of said animal,

b) contacting said animal with said compound, and

c) determining whether said compound affects cell death in said animal.

5. The method of claim 4, wherein step b) is carried out with said animal at an embryonic stage.

6. The method of claim 4, wherein said animal is a vertebrate.

7. The method of claim 6, wherein said vertebrate is a fish.

8. The method of claim 7, wherein said fish is a zebrafish.

9. A method of claim 6, wherein said determining step c) includes determining whether said compound affects cell death in a Rohon-Beard neuron.

10. A method of claim 9, wherein said determining step c) further includes determining whether said compound rescues a Rohon-Beard neuron that has been contacted with an agent which increases apoptosis.

11. A method of claim 4, wherein said determining step c) includes using an antibody to label a cell which undergoes cell death.

12. The method of claim 4, wherein said agent is staurosporine.

13. A method of testing a compound for the ability to affect cell death, said method comprising the steps of:

a) providing an osteichthes embryo which is translucent or transparent,

b) contacting said compound with said embryo, and

c) visually observing the pattern or extent of cell death in said embryo.

14. The method of claim 13, wherein said embryo is a zebrafish embryo.

15. The method of claim 13, wherein cells in said embryo undergoing programmed cell death are labeled in the living embryo for visualization microscopically.

16. The method of claim 15, wherein labeling is carried out by terminal deoxynucleotide transferase dUTP nick labeling.

17. A method for obtaining information on cellular processes, said method comprising the steps of:

a) providing a test and a control zebrafish embryo,

b) subjecting said test embryo to test conditions, and

c) visually observing differences in cells of the test and control embryos, said differences resulting from application of said test conditions.

18. The method of claim 17, wherein said test conditions include mutagenesis-inducing conditions.

19. The method of claim 17, wherein said test and control zebrafish embryos are pre-treated with an agent which affects programmed cell death.

20. The method of claim 17, wherein said test conditions include a test compound, and said observing step includes observing whether said test compound inhibits cell death in, or rescues, said test embryo.

21. The method of claim 20, wherein said observing step includes observing Rohon-Beard neurons.

22. The method of claim 19, wherein said agent is a protein kinase inhibitor.

23. The method of claim 22, wherein said protein kinase inhibitor is staurosporine.

24. The method of claim 17, wherein said test conditions include contacting said embryos with a cell death inhibitor.

25. The method of claim 24, wherein said observing includes observing neurons of said embryos, to determine whether neurons in said test embryo which are saved from cell death develop or function normally.

26. The method of claim 24, wherein said cell death inhibitor is a caspase inhibitor.

27. The method of claim 17, wherein the cellular process which is investigated is selected from the group consisting of:

a) neuronal cell function,

b) neuronal connectivity,

c) cell development,

d) tissue development, and

e) organ development.

28. A method of testing a compound for the ability to affect expression of a gene whose expression affects cell death, said method comprising the steps of:

(a) providing an osteichthes test embryo which is translucent or transparent, wherein said gene is expressed in said embryo, wherein said gene

either is not normally expressed in said osteichthes, or is normally expressed in said osteichthes at a lower level than in said test embryo,

(b) contacting said compound with said embryo, and

(c) visually observing the pattern or extent of cell death in said embryo.

29. The method of claim 28, wherein said osteichthes embryo is a zebrafish embryo.

30. The method of claim 28, wherein said gene is a eukaryotic gene encoding a protein which inhibits cell death.

31. The method of claim 30 wherein said gene encodes bcl-2, and is over-expressed in said embryo.

PCT

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(54) Title: *IN VIVO* APOPTOSIS SCREENING

(57) Abstract

A test animal for screening compounds for the ability to affect cell death, prepared by the process of: a) providing an animal embryo, b) contacting the embryo with a protein kinase inhibitor to increase apoptosis in cells of the animal; and methods of using the test animal.

PATENT
ATTORNEY DOCKET NO. 50015/013001

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am an original, first, and joint inventor that is claimed and for which a patent is sought on the invention entitled *in Vivo* Apoptosis Screening, the specification of which was filed on July 21, 1999 as Application Serial No. 09/355,004.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56.

FOREIGN PRIORITY RIGHTS: I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Serial Number	Filing Date	Priority Claimed?
United Kingdom	9701245.4	January 22, 1997	Yes
PCT	PCT/US98/01322	January 22, 1998	Yes

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D., Reg. No. 35,238, Kristina Bieker-Brady, Ph.D., Reg. No. 39,109, Susan M. Michaud, Ph.D., Reg. No. 42,885, Mary Rose Scozzafava, Ph.D., Reg. No.36,268, James D. DeCamp, Ph.D., Reg. No. 43,580, Sean J. Edman, Reg. No. 42,506, Timothy J. Douros, Reg. No. 41,716.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Signature:			Date:

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